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Enclosed is a copy of Priority Document 01200551.8 EP filed February 16, 2001 for the above referenced application.

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

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Pixel arrays

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Title: Pixel arrays

The invention relates to the detection of (bio)molecules or analogues thereof in micro-arrays and the supports used for said micro-arrays, in particular in methods for determining or testing binding of a first member molecule within an array or library of tentative first member binding molecules with a second member binding molecule, the first and second molecule each member of a binding pair and to enzyme-linked detection of said pair in high-density micro-array systems.

Interactions, or the formation of a specific binding pair, between binding molecules, which in general are bio-molecules, and their corresponding ligands, which in general are also bio-molecules are central to life. Cells often bear or contain receptor molecules that interact or bind with a hormone, a peptide, a drug, an antigen, an effector molecule or with another receptor molecule; enzymes bind with their substrate; antibody molecules bind with an antigen, nucleic acid with protein, and so on. By "interact or bind" it is meant that the binding molecule and ligand (or the functional parts thereof) approach each other within the range of molecular forces, and may influence each others properties. This approach takes the binding molecule and its ligand through various stages of molecular recognition comprising increasing degrees of intimacy and mutual effect: they bind and the two members form a pair.

Binding molecules have this binding ability because they comprise distinct binding sites allowing for the recognition of the ligand in question. The ligand, in turn, has a corresponding binding site, and only when the two binding sites can interact by -- essentially spatial -- complementarity, the two molecules can bind. Needless to say that, molecules having three dimensions, binding sites are often of a three dimensional nature, often one or more surface projections or protuberances of one binding site correspond to one or more pockets or depressions in the other, a three-dimensional lock-and-key arrangement, sometimes in an induced-fit variety.

Due to the central role binding molecules and their ligands play in life, there is an ever expanding interest in testing for or identification of the nature or characteristics of the binding site and the members of the binding pair of

molecules involved in such a site. Not only is one interested in the exact nature of the particular interaction between binding molecule and ligand in question, for example in order to replace or supplement binding molecules or ligands when needed; one is also interested in knowing approximating characteristics of the interaction, in order to find or design analogues, agonists, antagonists or other compounds mimicking a binding site or ligand involved.

Versatile and rapid methods to test for or identify binding pairs and its separate members are known. Most, if not all nucleic acid detection techniques, and molecular libraries using these, entail hybridisation of an essentially continuous nucleic acid stretch with a complementary nucleic acid strand, be it DNA, RNA or PNA. Protein and peptides are often detected using antibodies or derivatives or synthetic variants thereof. Arrays of biological molecules (micro-arrays) are currently used in standard techniques in many laboratories. Such micro-array-based detection generally comprises a method in which a member of a specific binding pair is detected by means of an optically detectable reaction. Different supports for the libraries comprising tentative or possible first members of the binding pair (be it nucleic acid, peptide, or of any other nature) are used but can roughly be divided in two types: porous surface and non-porous surfaces. An array or library of such first members is provided, in general spatially and/or addressable bound to such a support e.g. by spotting or gridding, and a second member, the detecting or specific binding molecule--which is now commonly directly or indirectly labelled with a marker molecule (such as a fluorescent compound) to facilitate optical detection--, of the aforementioned pair is than provided to detect the one(s) of the array of putative first members with which it can bind. Said second member can of course also be a nucleic acid, receptor molecule or antibody or the like. Binding of the second member thus identifies the first member because of its specific localisation on the support.

Porous surfaces (membranes, cellulose and paper) are probably the oldest in use: for example "dot blots" are widely used even nowadays. Even synthesis of macromolecules (e.g. nucleic acids or peptides) has been described on these porous matrixes. Paper was used as a relatively thick continuous porous matrix on which such first member constructs were synthesised spot wise. Binding pairs were generally identified by detection with directly or via indirectly enzyme-labelled probes, allowing increased sensitivity over the use of probes that were directly labelled with an optically detectable reporter molecule, such as a

fluorescent group. Disadvantage of these methods is that the density of spots in these matrixes is limited. This limitation is caused among other things by the diffusion in the matrix of the enzymatically changed substrate. To avoid this disadvantage of diffusion, and thus inaccurate localisation of a binding pair, in the field of peptide synthesis, methods are described on polyethylene pins (Geysen 1983), or in polypropylene wells (Slootstra, 1995; 1997). However, these "early" methods all have the disadvantage that no high density arrays (approximately up to not more than 10-20 spots/cm2) can be facilitated due to various reasons.

Limited spot density in the arrays is the reason why more recently non-porous surfaces are more widely used. Especially in field of genomics, nowadays, huge arrays of polynucleotide sequences are spotted on a variety of surfaces, most of the time on glass slides covered with different coatings (US patent 6015880, US patent 5700637). Array densities of 1000 spots/cm2 are easily possible. Even higher densities are possible when the biomolecules (poly nucleotide sequences) are synthesized in-situ (US patent 5871928). For eaxample, in a traditional gene expression assay, designed to profile the expression of many genes in parallel, mRNA is prepared from two different tissue types (e.g. normal and diseased samples). The isolated mRNA represents a snapshot of the current state of expression within the cells. The mRNA is converted to DNA via a first-strand cDNA labelling reaction. After target DNA is deposited onto coated glass slides and directly labelled cDNA probes are hybridized to the arrays. Hybridized arrays are imaged using an array scanner and the results are examined for differences in expression levels using several image and data analysis software tools. Recently for these purposes a more intricate porous support surface has been described (WO 00/56934), named continuous porous matrix arrays. On microscope slides a continuous slab of polyacrylamide is formed (for example 20um thick, whereby a thin continuous porous matrix (hydrogel) is combined with a non porous surface (glass).

Detection of specific binding pairs on or in said high-density supports is in general achieved with directly labelled probes comprising optically detectable (in general fluorescent) nucleotides or antibodies. These are in general of high sensitivity, have low non-specific binding and high photo stability. Labelled nucleotides are widely used for labelling DNA and RNA probes especially for

multicolour analysis in micro-arrays, but also for FISH, chromosome identification, whole chromosome painting, karyotyping and gene mapping. Labelled nucleotides are available in a range of bright, intense colours with narrow emission bands ideal for multiplexing within a single sample. For protein or peptide detection fewer fluorescently labelled probes are available, since the field of protein or peptide based high-density micro-array systems has not yet as well developed as micro-arrays based on nucleic acid detection.

The invention combines the advantages of high density arraying (testing a lot of binding events in one go) and enzyme-linked assays (very sensitive) allowing to detect more binding pairs more rapidly. Micro-array systems are provided herein that allow to work with enzyme-linked assays to detect the molecule of interest on high-density supports. Such testing high densities of constructs on a solid support in a enzyme-linked assay is provided by the invention, wherein for instance a first member is provided to or synthesised on a surface of the support in a density of, for instance, at least 25 or preferably at least 50, but more advantageously preferably at least 100, or more, such as 200-500 or even 1000 spots per square centimeter. Said first binding pair members are for example spotted or gridded, in a positionally or spatially addressable way, giving so many different constructs or first member molecules on the support with which a second member or binding molecule can react. Of course, spots can overlap, as long as the constituting collection of first member molecules are spatially addressable and distinct. Spotting can, for instance, be done using piezo drop-on-demand technology, or by using miniature solenoid valves. Gridding can, for instance, be done using a set of individual needles that pick up sub-microliter amounts of segment solution from a microtiter plate, containing solutions comprising the first members. When testing peptides, after the linking reaction, subsequent deprotection and extensive washing of the support to remove uncoupled peptide gives at least a peptide construct density as large as 25 to 50, or even 100 to 200, or up to 500 to 1000 spots per cm^2. This density allows to screen a great many possible peptide constructs of said proteins for binding with an antibody. For example: in a preferred embodiment 25000 to 100.000 constructs are made on 1000 cm^2, typically the surface is than screened for binding in enzyme-linked assay—be it directly or indirectly-- wherein a fluorescent substrate is generated with 100 ml of enzyme-labelled probe solution,

containing 1 - 10 µg of probe/ml and subsequent development of an optically detectable substrate with established techniques. The invention thus provides a method for determining binding of a first member molecule within an library of tentative first member binding molecules with a second member binding molecule comprising providing a support with a library of spots of said tentative first member binding molecules in a density of at least 25 spots per square centimetre and detecting said binding in an enzyme-linked assay, preferable wherein said enzyme-linked-assay comprises the production of fluorescent or chemiluminescent substrate. Fluorescent substrates can be produced with a host of enzyme systems such as hors-radish-peroxidase, alkaline phosphatase or other substrate-enzyme systems that are known in the art.

For example, indirect or direct fluorescence detection allocates antibody binding constructs. Direct fluorescence detection with confocal scanning detection methods for example allows antibody detection on spots generated with droplets peptide-solution in the sub-nanoliter range, making even higher construct densities feasible. Of course, nucleic acid libraries can be made in a similar fashion, using enzyme-labelled nucleic acid probes.

Furthermore, the invention provides a support for a micro-array suitable for testing binding of a first member molecule within an array or library of tentative first member binding molecules with a second member binding molecule said support provided with a surface wherein patches are interspersed within areas that are materially distinct from said patches.

Herewith, the invention provides a method for determining binding of a first member molecule within an library of tentative first member binding molecules with a second member binding molecule comprising providing a support with a library of spots of said tentative first member binding molecules, detecting said binding in an enzyme-linked assay and providing for limited, minimalised or restricted diffusion of an optically detectable marker molecule. Now that diffusion is limited, the enzymatic reaction and the deposit or localisation of the resulting (optically) detectable marker molecules can be determined with much more precision, allowing much higher densities than with previous micro-arrays using enzyme-linked-detection was deemed feasible. In particular, the invention provides a method wherein said diffusion is limited by providing the surface of said support with a support surface wherein surface

patches are interspersed within surface areas that are materially distinct from said patches.

In particular, the invention provides a support (herein also called a discontinuous matrix array or pixel array) wherein the support surface material is of a varied or discontinuous nature as regards to hydrophilicity. In one embodiment of such a support for a high-density micro-array as provided herein, patches of relative hydrophilicity are preferably interspersed with areas of relative hydrophobicity. Of course there need not be a sharp border between patches and the surrounding area, it is sufficient when distinct material differences or discontinuities exist between the centre of a patch and the middle line of a surrounding area, whereby there is a more or less gradual material change in between. Patches and surrounding areas may be in strict matrix or grid format, but this is not necessary. Patches are in general somewhat, but preferably at least one or two dimensions smaller than the size of the circumference of the positioned droplets or spots of first member molecules that in a later phase will be provided to the support surface, that is preferably at least 3-5, and more preferably at least 10-20 of such e.g. hydrophilic patches fit within the circumference of a later spotted solution of a first member, be it nucleic acid or peptide or any other (bio-)molecule or combination thereof. Patches resemble pixels that, after a marker molecule has attached to a specific binding pair, create the optically detectable image whereby a spot with a collection of first member molecules bound to second member molecules is detected. Of course, a one-to-one fit of pixel or patch to droplet or spot is also feasible, even when the patch is larger than a spot, but not necessary. Neither is it necessary to apply or provide for the patches in an overly regular pattern. When a droplet or spot is provided, the interspersed hydrophobic character of the support surface will limit the diffusion of any aqueous solution, and thus also, again in a later phase, the diffusion of a solution of an optically detectable substrate (be it as precipitate or as solution) formed after the enzymatic reaction that took place where a first member is bound to a second member of a binding pair, whereby the presence of the relatively hydrophilic patch or patches within said droplet or spot circumference allows said substrate to be positioned or detectable at all. The preferred patches as provided herein may also be described as pixels within the spot(s) where finally the optically detectable or fluorescent substrate will be located. Of course, if so desired patches may be hydrophobic

where the surrounding area is relatively hydrophilic, when for example solutions or (optically detectable) markers are tested of a more hydrophobic nature.

In a preferred embodiment, said support as provided herein comprises at least a spot or dot (e.g. a collection of first member molecules such as a nucleic acid or peptide construct) density as large as 25 or 50, or even 100, 200, or up to 500 or even 1000 spots per cm^2, preferably wherein said spots or dots are positionally or spatially addressable, each of said spot or dot covering at least one patch, but preferably from 3-5, or even from 5-15 or more patches or pixels.

Hydrophilic patch size can be modified by selecting the appropriate support material, such as polyethylene or polypropylene or another relatively hydrophobic plastic material, to begin with, or by providing it with patches in the desired size, e.g. by utilizing print technology. Below, a support surface is produced from a relatively hydrophobic polypropylene surface upon which grafts are provided that form the relatively hydrophilic patches. Preferred is to make the grafts with polyacrylic acid, which has an excellently suitable hydrophilic nature, allowing testing under physiological circumstances. Patch size can be influenced by selecting the appropriate roughness of a polyethylene or polypropylene starting material, said roughness can also be modulated by sanding or polishing , or by any other mechanical (printing) or chemical (etching) method to modulated a surface on which the hydrophilic patches are to be generated. Of course, the smaller the hydrophilic patch size is, the smaller the droplets to be applied can be, preferably up to the size where at least one patch falls within the circumference of the droplets applied.

The invention also provides a method for determining binding of a first member molecule within an library of tentative first member binding molecules with a second member binding molecule comprising use of a support according to the invention, in particular a method comprising providing said support with spots comprising said tentative first member binding molecules, providing a second member binding molecule and detecting binding of a first member molecule with said second member binding molecule.

Preferably, said binding is detected with an optically detectable marker for example wherein said marker comprises a fluorophore, directly or indirectly labelled to a probe such as a nucleic acid or antibody, thus allowing a support according to the invention to be used in any type of micro-array; prevention of diffusion is always welcome to avoid or circumvent problems such as signal

overload, however, in a preferred embodiment, the invention provided a method wherein binding pairs are detected via enzyme-linked-assay techniques, where otherwise diffusion or leakage would be much harder to overcome, the further advantage being that enzymetic detection is much more sensitive, thereby allowing to include less copies of tentative first member molecules to be spotted in one spot, thus in general decreasing spot-size, thus allowing to increase spot density, without having to give in on sensitivity. Enzymatic detection can be up to 10-1000 times more sensitive as detection of directly labelled probes.

Suitable enzyme-substrate combinations and methods for use in a method according to the invention are for example found with US4931223 wherein processes are disclosed in which light of different wavelengths is simultaneously released from two or more enzymatically decomposable chemiluminescent 1,2-dioxetane compounds, said compounds being configured, by means of the inclusion of a different light emitting fluorophore in each of them, to each emit light of said different wavelengths, by decomposing each of said compounds by means of a different enzyme. Also, Bronstein et al. BioTechniques 12 #5 (May 1992) pp. 748-753 "Improved Chemiluminescent Western Blotting Procedure" suggests an assay method in which a member of a specific binding pair is detected by means of an optically detectable reaction which includes the reaction, with an enzyme, of a dioxetane so that the enzyme cleaves an enzyme-cleavable group from the dioxetane to form a negatively charged substituent bonded to the dioxetane, the negatively charged substituent causing the dioxetane to decompose to form a luminescent substance. Cano et al. J. App. Bacteriology 72 (1992)provided an example of nucleic acid hybridization with a fluorescent alkaline phosphatase substrate, which advantagously can be used in the invention as well, and Evangelista et al. Anal. Biochem. 203 (1992) teaches alkyl-and aryl-substituted salicyl phosphates as detection reagents in enzyme-amplified fluorescence DNA hybridization assays. In the detailed description herein use is made of a fluorescent substrate for alkaline phosphatase-based detection of protein blots, for use with fluorescence scanning equipment such as Molecular Dynamics FluorImager or Storm instruments, generally known as Vistra ECF and generally only deemed suitable for use in Western blotting, dot and slot blotting applications. The enzymatic reaction of alkaline phosphatase dephosphorylates said ECF substrate to produce a fluorescent product which is, as shown herein, detectable in a method according to the invention. However,

9. A method according to claim 8 wherein said binding is detected with an optically detectable marker.

10. A method according to claim 9 wherein said marker comprises a fluorophore.

11. A method according to claim 10 wherein said binding is detected in an enzyme-linked-assay.

12. A method according to claim 11 wherein said enzyme-linked-assay comprises the production of fluorescent substrate.

13. A method according to claim 12 wherein said enzyme comprises alkaline phosphatase.

14. A method for determining binding of a first member molecule within an library of tentative first member binding molecules with a second member binding molecule comprising providing a support with a library of spots of said tentative first member binding molecules in a density of at least 25 spots per square centimetre and detecting said binding in an enzyme-linked assay.

15. A method according to claim 14 wherein said enzyme-linked-assay comprises the production of fluorescent substrate.

16. A method according to claim 15 wherein said enzyme comprises alkaline phosphatase.

17. A method for determining binding of a first member molecule within an library of tentative first member binding molecules with a second member binding molecule comprising providing a support with a library of spots of said tentative first member binding molecules, detecting said binding in an enzyme-linked assay and providing for limited diffusion of an optically detectable marker molecule.

Claims

1. A support for a micro-array suitable for determining binding of a first member molecule within an library of spots of tentative first member binding molecules with a second member binding molecule said support provided with a support surface wherein surface patches are interspersed within surface areas that are materially distinct from said patches.

2. A support according to claim 1 wherein said patches are relatively hydrophilic whereas said areas are relatively hydrophobic.

3. A support according to claim 1 or 2 wherein the surface of said areas essentially comprise relatively hydrophobic polypropylene whereas the surface of said patches essentially comprise polypropylene provided with a relatively hydrophilic material.

4. A support according to claim 3 wherein said relatively hydrophilic material comprises polyacrylic acid.

5. A support according to any one of claims 1 to 4 provided with a library of tentative first member binding molecules in spatially addressable spots.

6. A support according to anyone of claims 1 to 5 comprising a spot density of larger than 25 spots per cm^2

7. A method for determining binding of a first member molecule within an library of tentative first member binding molecules with a second member binding molecule comprising use of a support according to anyone of claims 1 to 6.

8. A method according to claim 7 comprising providing said support with spots comprising said tentative first member binding molecules, providing a second member binding molecule and detecting binding of a first member molecule with said second member binding molecule.

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described in example 3. In this scan all sequences are subsequently shifted simultanuously through the complete protein sequence to obtain the complete library. B) Working example of obtained wityh anti-hFSH monoclonal antibody-02. C) Binding values and list of peptides coupled onto each other. D)One square in full detail. The peptide br-CKELVYETVRVPG was coupled to the cysteine of card 06. To this card peptides 1 to 36 were spotted with gridding pins. The binding values are shown below. Chemistry in short: Polypropylene (PP) surface was gamma irradiated (in this case 50kGy) in the presence of $CuSO_4$ and (in this case 12%) acrylic acid. Carboxylic acid functionalized PP was treated with Boc-HMDA/DCC/HOBt and subsequent treatment with trifluoracetic acid (TFA) yielded a surface with amino groups. To this amino group functionalized PP surface, N-Fmoc-S-trityl-L-cysteine (Fmoc-Cys-(Trt)-OH) was coupled using DCC and HOBt. Subsequently the Fmoc group was removed, followed by acetylation of aminogroup. Treatment of the surface with TFA (with triethylsilan and water as scavangers) yielded a thiol functionalized surface. Bromoacetyl (or other thiol reactive) containing peptides were allowed to react with the thiol groups of the PPsurface in 0.015M $NaHCO_3$ (pH 7-8, overnight reaction). Subsequently the -StBu groups (of the S-t-butylthio protected Cys residues) of the coupled peptides were removed using $NaBH_4$ (14mg/ml in 0.015M $NaHCO_3$ pH 7-8, 30min, 30 C), generating new thiol groups in the peptides. A second set of Bromoacetyl (or other thiol reactive) containing peptides were then allowed to couple to the first set, making peptide constructs. This proces can be repeated several times using different sets of bromoacetylated peptides.

Figure 8. Storm fluorescence signals of the binding of Glu-ox to Mab GO1 on 3 different grafts using five different gridding pins

pins). Four different peptide concentrations were spotted on three different grafts: 12/50 Ac, 9/30 Ac and 6/12Ac grafts.

Figure 4. shows maximal fluorescent signals of the spots as detected by the Storm of the binding of Mab GO1 to the peptide nr 1, 2, 3 and 4 on graft 6/12Ac using four different peptide concentrations and five different gridding pins. Petide concentrations: Figure 4a: 1mg/ml; 4b: 0.2mg/ml; 4c: 0.04mg/ml and 4d: 0.008mg/ml.

Figure 5. shows maximal fluorescent signals of the spots as detected by the Storm of the binding of Mab GO1 to peptides nr 1, 2, 3 and 4 (peptide concentration 0.2 mg/ml) on graft 6/12Ac, 9/30Ac and 12/50Ac. Figure 4a: graft 6/12Ac; 4b: graft 9/30Ac; 4c: graft 12/50Ac.

Fig. 6. A) Schematic presentation of a head-to-tail complete matrix scan. 12345678901 and ABCDEFGHIJK represent sequences derived from a protein. And, Schematic presentation of a tail-to-tail complete matrix scan. This scan is similar to the scan shown in fig. 4, however, the cysteine residue is positioned at the N-terminus of the second building block, leading to a reversed or tail-to-tail orientation of both building blocks. Both sequences are linked as described previously. In this scan both sequences are shifted independently through the complete protein sequence, generating a library of all possible sequence combinations. B) List of all peptides (derived from hFSH) containing an N-terminal bromoacetamide group. C) List of all peptides (derived from hFSH) containing a C- or N-terminal cysteine. D) Complete matrix scan, i.e. after coupling of ALL listed in B sequences to ALL listed in C sequences, eexemplified by cards 145-155 and a full picture of all binding values of all ca. 40.000 peptides (below).

Fig. 7. A) Schematic presentation of a multi-building block scan. 12345678901 (building block 1), NOPQRSTUVWXY (building block 2) and BCDEFGHIJKLM (building block 3) represent successive sequences derived from a protein. Building blocks were linked via a thioether bridge, formed by reaction of a free thiol function of a C-terminal cysteine residue (C) in building block 1 and a bromoacetamide group ($) at the N-terminus of building block 2 and so on, as

first building block. This means that every combination of, for instance, undecapeptides from the protein sequence is being synthesised on a seperate, known, position of the solid support.

Example-3b (type II): tail-to-tail matrix-scan.
This is the same scan as the complete matrix scan from example 2a, however, in this scan the cysteine of the second building block is located at its N-terminus, providing a reversed or tail-to-tail orientation of both building blocks in the construct as also shown in figure 6A.

Both example 3a and 3b are illustrated in Figs 6B, 6C and 6D.

Example-4: Multi building block scan.
In this example a thiol fuction is introduced on an amino-functionalised solid support. This can be made by a direct reaction of the amino groups with, for instance, iminothiolane, or by coupling of Fmoc-Cys(Trt)-OH, followed by Fmoc cleavage using piperidine, acetylation, and trityl deprotection using TFA/scavenger mixtures. This thiol-functionalised solid support can be reacted with, for instance, a bromoacetamide-peptide, containing a protected cysteine residue. After coupling of the first peptide, the cysteine can be deprotected, using, for instance, a TFA/scavenger mixture. The formed free thiol group can be used to couple a second bromoacetamide-peptide, again containing a protected cysteine. This procedure can be repeated to make multi-building block constructs. Several types of scans, as described in the other examples, can be used in combination with this multi building block scan. In fig. 7A an example is shown for a three multi building block scan. An working example with two building block scan is illustrated in 7B, 7C and 7D.

Figure 1. Surface structure of polyacrylic acid grafted PP.

Figure 2. ECF-substrate wettability of different surfaces.

Figure 3. Storm fluorescence signals of the binding of peptide nr 1,2,3 and 4 (y-axis) to Mab GO1 using five different gridding pins (on X-axis diameter gridding

thiol reactive bromacetamide group on the support, the amino group functionalised support was treated with bromoacetic acid using DCC or DCC/HOBt.

Glucose oxidase containing thiol-groups (Glu-ox-SH) was able to couple to the bromo functionalised surface. Thiol groups on Glucose oxidase (Glu-ox;1mg/ml) were introduced in 0.16M borate buffer (pH8) using 2-iminothiolane (5 times molar excess 2-iminothiolane over Glu-ox ;45 min; room temperature). Glu-ox-SH was spotted on the bromo functionalised surface using gridding pins (Genomic Solutions) with different diameters (1.5mm, 0.8mm, 0.6mm, 0.4mm and 0.25mm). Concentration of Glu-ox-SH was 0.25mg/ml. When aliquots of Glu-ox-SH solutions (in phosphate buffered saline =PBS , 1mM Titriplex=EDTA at pH7) were dispensed on the support using the grdding pins, the coupling of the bromo group of the surface to the thiol group of Glu-ox-SH was achieved in a humid chamber(overnight reaction). Extensive washing removed uncoupled Glu-ox-SH.

Binding of an antibody (Mab GO1) to Glu-ox was detected using a method which made use of a fluorescent product: The whole PP support containing th Glu-ox functionalised areas was incubated with the antibody GO1 (5ug/ml). After washing a subsequent incubation of a second anti mouse antibody conjugated to alkaline phosphatase introduces, after binding of the Mab to Glu-ox, the enzyme alkaline phosphatase at the Glu-ox functionalised surface (spots). After washing the bound enzyme caused fluorescent product signals at the peptide functionalised surfaces when Vistra ECF substrate (Amersham Pharmacia Biotech)(excess substrate was removed) was introduced. Fluorescent product signals could be quantified on a Storm (Molecular Dynamics) in blue fluorescent mode. Figure 6 shows the Storm fluorescent signals of the binding Glu-ox to Mab GO1 using five different gridding pins and three different grafts.

Example-3a: head-to-tail matrix-scan.

In a complete matrix-scan the N-terminal sequence of, for instance, sequence [1 - 11] of a protein, is linked as a building block with each overlapping peptide sequence of a complete scan of the same protein as shown in figure 6A. Next, sequence [2 - 12] is linked with the same set of overlapping sequences and so on. The link can be formed, for instance, by reaction of a cysteine at the C-terminus of the second building block with a bromoacetamide modified N-terminus of the

aliquots of peptide solutions (in bicarbonate buffer at about pH7-8) were dispensed on the support using the gridding pins, the coupling of the bromo group on the surface to the thiol group of the peptide was achieved in a humid chamber (overnight reaction). Extensive washing removed uncoupled peptide. Peptides used are: GCASLQGMDTCGK (Nr1), CAFKQGVDTCGK (Nr2) APDPFQGVDTCGK (Nr3), and GCAPDPFQGVDTCGK (Nr4). From surface plasmon resonance (SPR) measurements affinity constants are known with antibody Mab GO1: Nr1 kD=<10-3; Nr2 kD=2.10-6; Nr3 kD=3.10-7 and Nr4 kD=6.10-8.

Binding of the antibody to the peptides was detected using a method, which made use of a fluorescent product: The whole PP support containing the peptide functionalised areas was incubated with the antibody (Mab GO1 5ug/ml, incubation overnight). After washing a subsequent incubation of a second anti mouse antibody conjugated to alkaline phosphatase, introduce, after binding of the Mab to the peptide, the enzyme alkaline phosphatase at the peptide functionalised surface (spots). After washing the bound enzyme caused fluorescent product signals at the peptide functionalised surfaces when a thin film of a Vistra ECF substrate (Amersham Pharmacia Biotech) solution was added to the surface (excess substrate was removed). Fluorescent product signals could be quantified on a Storm (Molecular Dynamics) in blue fluorescent mode. Figure 3 shows the Storm fluorescent signals of the binding of the peptides Nr 1,2,3 and 4 to Mab GO1 using five different gridding pins and four different peptide concentrations on 3 different grafts. Figs 4A,B,C,D show the maximal fluorescent signals of the spots on graft 6/12Ac. Figure 5 shows the maximal fluorescent signals of peptides Nr 1,2,3 and 4 spotted with 0.2 mg/ml on graft 6/12Ac, 9/30Ac and 12/50Ac.

Example 2. Glucoase Oxidase. A polypropylene (PP) support (EVACAST 1070 N16; Vink Kunststoffen BV) was grafted with polyacrylic acid. The solid support was irradiated in the presence of 6% acrylic acid solution in water, containing CuSO4 using gamma radiation at a dose of 12kGy. The grafted solid support containing carboxylic acid groups was functionalised with amino groups via coupling of T-butyloxycarbonylhexamethylenediamine (Boc-HMDA) using dicyclohexylcarbodiimide (DCC) with N-hydroxybenztriazole (HOBt) and subsequent cleavage of the Boc groups using trifluoracetic acid. To introduce a

in a later stage removed from the surface, dye development does not suffer from diffusion problems. This phenomenon is caused by the valley/hill or hydrophobic /hydrophilic construction of the surface in combination with excellent wettability properties of polyacrylic acid matrix. Figure 2 shows the Vistra ECF (2'(2-benzthiazoyl}-6'-hydroxy-benzthiozole phosphate bis-(2-amino-2-methyl-1,3-propanediol) salt; Amersham Pharmacia Biotech) substrate wettability of i) with and ii) without poly acrylic acid grafted PP (EVACAST 1070 N16; Vink Kunststoffen BV) and iii) CMT-glass slides (Corning) as detected on a Storm Fluorimager (Molecular Dynamics). Although the, with polyacrylic acid grafted PP-EVACAST surface is not continuous occupied with porous (polyacrylic acid grafts) material, the Storm Fluorimager does not detect irregular surface patterns. This in contrast to ungrafted PP-EVACAST or CMT-glass slides.

Examples of use

Example-1: A polypropylene (PP) support (EVACAST 1070 N16; Vink Kunststoffen BV) was grafted with acrylic acid to introduce polyacrylic acid grafts on the PP surface. In this case: The solid PP support was irradiated in the presence of 6%, 9% or 12% acrylic acid solutions in water, containing $CuSO_4$ using gamma radiation at a dose of 12, 30 or 50kGy (combinations: 6% acrylic acid and 12kGy = 6/12Ac; 9% acrylic acid with 30 kGy = 9/30Ac and 12% acrylic acid with 50 kGy = 12/50Ac). The grafted solid support containing carboxylic acid groups was functionalised with amino groups via coupling of T-butyloxycarbonylhexamethylenediamine (Boc-HMDA) using dicyclohexylcarbodiimide (DCC) with N-hydroxybenztriazole (HOBt) and subsequent cleavage of the Boc groups using trifluoracetic acid. To introduce a thiol reactive bromacetamide group on the support, the amino group functionalised support was treated with bromoacetic acid using DCC or DCC/HOBt.

Peptides containing cysteine residues were able to couple to the bromo functionalised surface via the thiol group of the cysteine residues forming a stable thioether bond: Peptides were spotted on the bromo functionalised surface using gridding pins (Genomic Solutions) with different diameters (1.5mm, 0.8mm, 0.6mm, 0.4mm and 0.25mm). Solutions with different concentrations of peptide were used (1 mg/ml, 0.2 mg/ml, 0.04 mg/ml and 0.008 mg/ml). When

Rough polypropylene (PP) supports are commercial available and are widely used as not shiny material in all sorts of applications. This rough PP appeared to be an ideal template for attaching polyacrylic acid grafts. For example, microscope viewing of PP (EVACAST 1070 N16; Vink Kunststoffen BV) surface reveal rounded elevations (hills) separated by tiny depressions (valleys). See figure 1. The PP surface on top of the hills is relatively rough compared to the surface of valleys between the hills. Rough surface appeared to be a good scaffold for attaching grafts whereas the depressions accept grafts less readily. So during grafting procedures using gamma irradiation, the graft is not regular dispersed along the surface but is deposited in patches surrounded by materially different areas corresponding to the depressions in the material. For example using CuSO4 and acrylic acid during grafting most of the polyacrylic acid polymers are grafted on the top of the elevations, less in the depressions (see figure 1). As such on the grafted PPsurface support a more-or-less regular pattern of hydrophylic (polyacrylic acid grafts) patches and relatively hydrophobic (places without or less polyacrylic acid grafts) areas are present. The pattern of hydrophilic (normally hydrophilic matrixes causes severe diffussion) and hydrofobic areas (blocks diffusion) diminish diffusion especially when the patches are smaller then the droplet size of dispensed material. Although the surface of the PP is not completely covered with a homogenous graft high loadings of peptide /cm2 are possible, due to the relatively high surface occupation of the polyacrylic acid grafts on these PP surfaces. It is obvious that in the above described setup thicker grafts can carry higher peptide loadings but will suffer from more diffusion problems of dispensed material because of the growing occupation of grafted surface. However, the material can be made to suit various needs as regard to loading versus diffusion.

Enzyme-linked assays make use of substrates which are converted by the enzyme in products that or precipitate in situ or are water soluble. A drawback of precipitating products is the not-reuseability of the system caused by insolubility of the precipitated material during cleaning. Preferable is the set up which make use of non precipitating products, in particular not precipitating products which are fluorescent, because of the ease of detection by modern fluorescent signal detecting applications. When substrates (developing soluble products) are put on the surface, preferably where excess of substrate material is

not only alkaline phosphatase detection based is provided herein, the invention also provides a method according to the invention wherein a substrate for evaluating glycosidic enzymes comprising a fluorescein derivative such as known from US5208148 is used, which bears a lypophilic character and therefor will preferably reside in hydrofobic areas of the surface. Furthermore, the invention provides a synthetic molecule comprising a binding site(i.e. located on the detected first member molecule or derivatives thereof) or a binding molecule comprising a binding site identifiable or obtainable by a method according to the invention. Furthermore, use of a support or a method according to the invention for identifying or obtaining a synthetic molecule comprising a binding site or for identifying or obtaining a binding molecule capable of binding to a binding site is provided and the use of such an obtained molecule for interfering with or effecting binding to a binding molecule. The invention is further explained in the detailed description herein without limiting it.

Detailed description

By way of example mostly peptide related technology is described but the invention is just as well applicable to nucleic acid or other biomolecule detection. Conventional Pepscan methods make use of pins (Geysen et al) or wells (Slootstra et al). Polyacrylic acid grafts or other acrylic grafts on the polyethylene pins or in the polypropylene wells were used as carriers of peptides. Due to the high peptide loadings (each other carbon atom of the polymer can in theory carry a peptide) tested in an ELISA format extreme low binding -interactions of a peptide to an antibody can be detected (detection of kD <3 M are possible. In this system the interactions were always separated physically, í.e. by walls of wells. Technically miniturisation of this concept stops in practice at approximately 10 wells/cm2 due to the limitations of conventional (siringe /needle) liquid handling technics. When the set-up is miniaturised, it is desirable to keep the two strongholds (high peptide loadings in combination with enzym-linked detection methods) intact.

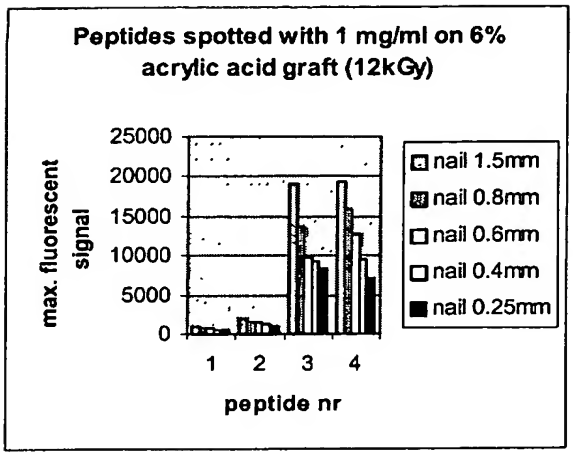


Figure 4a.

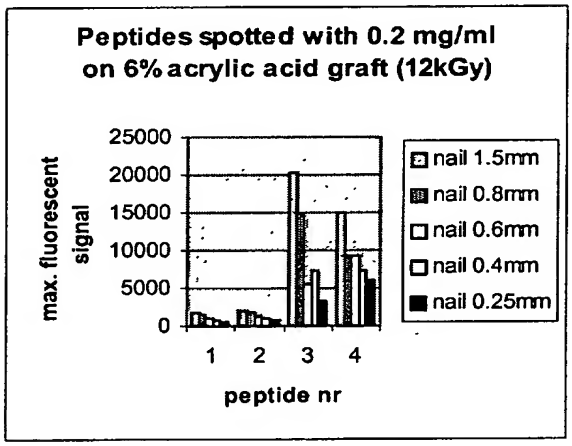


Figure 4b.

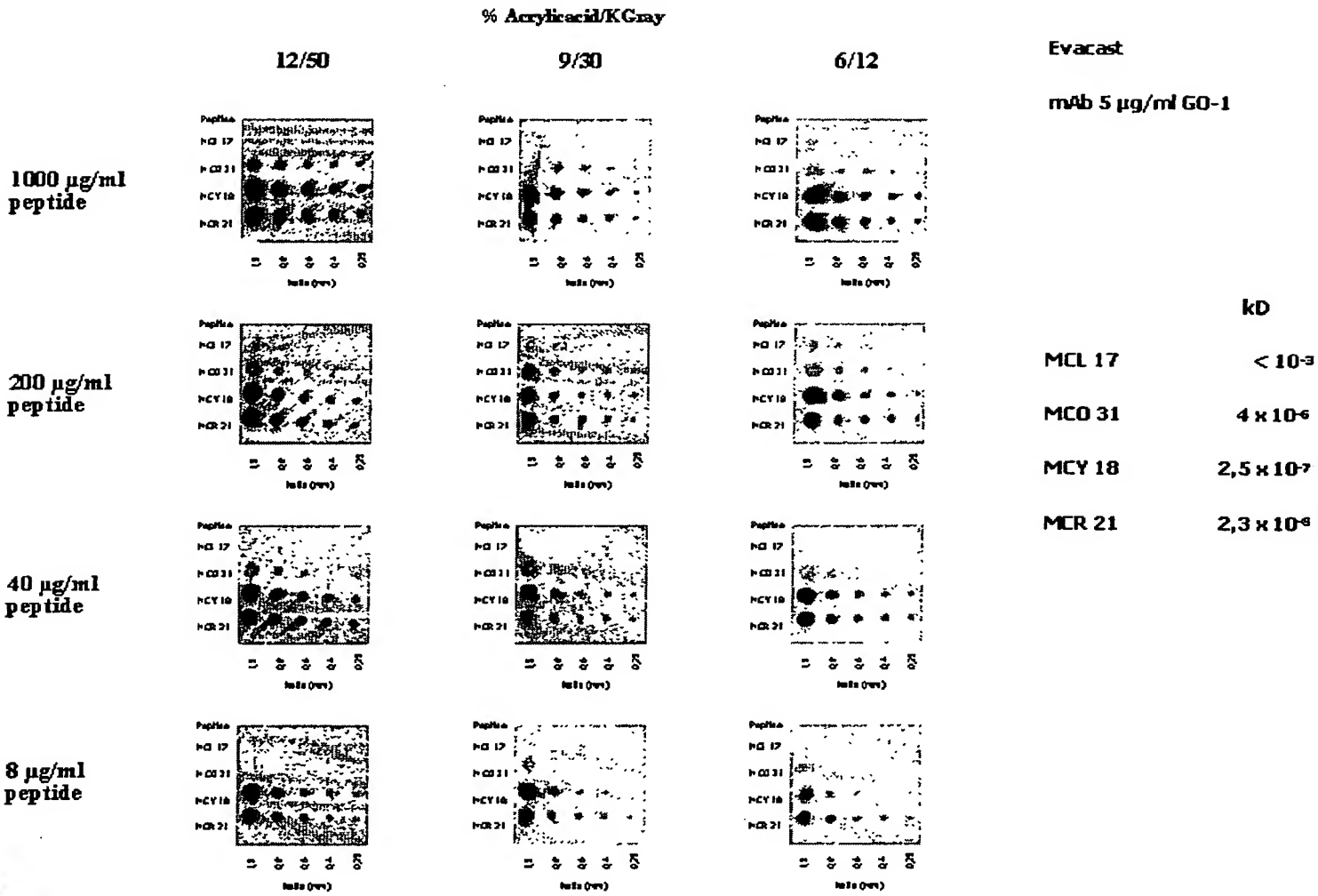

% Acrylicacid/KCgay
12/50
9/30
6/12
Evacast
mAb 5 µg/ml GO-1
1000 µg/ml peptide
200 µg/ml peptide
40 µg/ml peptide
8 µg/ml peptide
kD
MCL 17 < 10-3
MCO 31 4 x 10-6
MCY 18 2,5 x 10-7
MCR 21 2,3 x 10-8

Fig. 3.

Figure 2

Fig. 2.

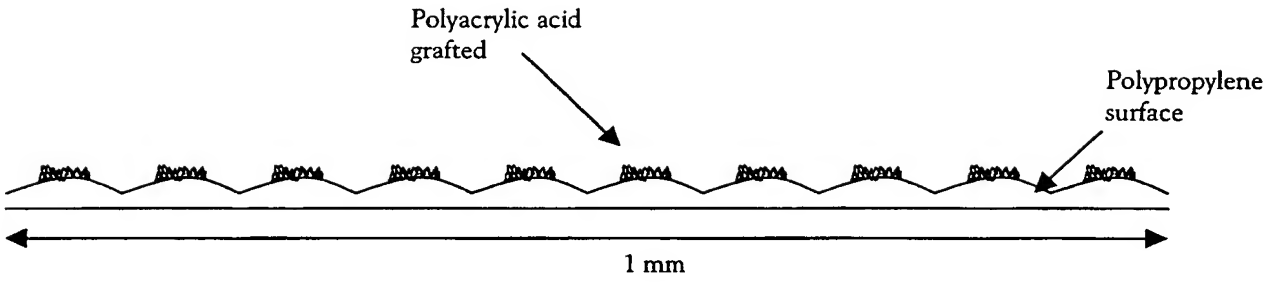


Fig. 1.

Abstract

The invention relates to the detection of biomolecules or analogs thereof in micro-arrays and the supports used for said micro-arrays, in particular in methods for determining or testing binding of a first member molecule within an array or library of tentative first member binding molecules for binding with a second member binding molecule. The invention provides a support for a micro-array suitable for determining binding of a first member molecule within an library of spots of tentative first member binding molecules with a second member binding molecule said support provided with a support surface wherein surface patches are interspersed within surface areas that are materially distinct from said patches.

27. Use of a molecule according to claim 24, 25 or 26 for interfering with or effecting binding to a binding molecule.

18. A method according to claim 17 wherein said diffusion is limited by providing the surface of said support with a support surface wherein surface patches are interspersed within surface areas that are materially distinct from said patches.

19. A method according to claim 18 wherein said patches are relatively hydrophilic whereas said areas are relatively hydrophobic.

20. A method according to claim 18 or 19 wherein the surface of said areas essentially comprise relatively hydrophobic polypropylene whereas the surface of said patches essentially comprise polypropylene provided with a relatively hydrophilic material.

21 A method according to claim 20 wherein said relatively hydrophilic material comprises polyacrylic acid.

22. A method according to any one of claims 14 to 21 wherein said library of spots is spatially addressable.

23. A synthetic molecule comprising a binding site identifiable or obtainable by a method according to anyone of claims 7 to 22.

24. A binding molecule comprising a binding site identifiable or obtainable by a method according to claim 7 to 22.

25. Use of a support according to anyone of claims 1 to 6 or a method according to claim 7 to 22 for identifying or obtaining a synthetic molecule comprising a binding site.

26. Use of a support according to anyone of claims 1 to 6 or a method according to claim 7 to 22 for identifying or obtaining a binding molecule capable of binding to a binding site.

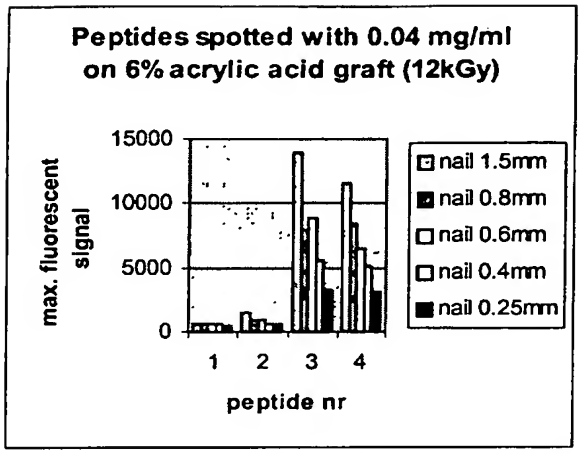


Figure 4c.

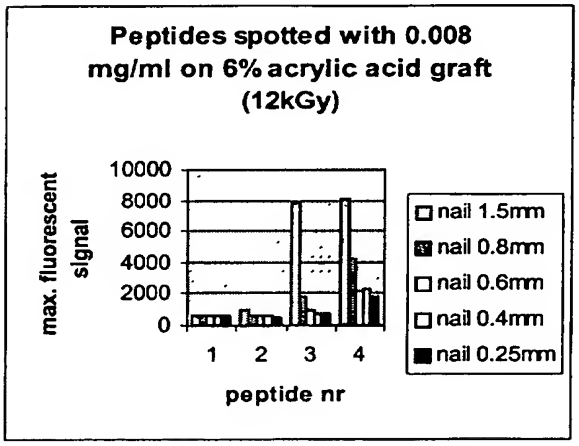


Figure 4d.

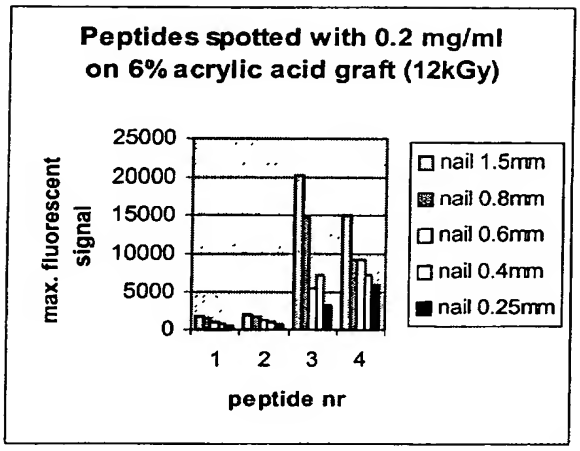


Figure 5a.

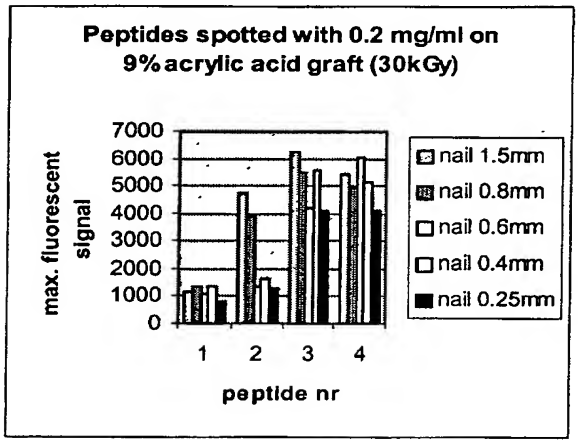


Figure 5b.

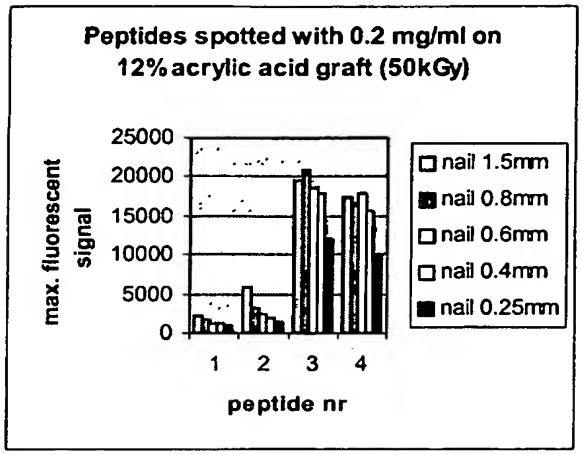

Peptides spotted with 0.2 mg/ml on 12% acrylic acid graft (50kGy)
max. fluorescent signal
25000
20000
15000
10000
5000
0
1
2
3
4
peptide nr
nail 1.5mm
nail 0.8mm
nail 0.6mm
nail 0.4mm
nail 0.25mm

Figure 5c.

12345678901C$ABCDEFGHIJK-Solid Support
12345678901C$BCDEFGHIJKL-Solid Support
12345678901C$CDEFGHIJKLM-Solid Support
... and so on
23456789012C$ABCDEFGHIJK-Solid Support
23456789012C$BCDEFGHIJKL-Solid Support
23456789012C$CDEFGHIJKLM-Solid Support
... and so on

or

C12345678901$ABCDEFGHIJK-Solid Support
C12345678901$BCDEFGHIJKL-Solid Support
C12345678901$CDEFGHIJKLM-Solid Support
... and so on.
C23456789012$ABCDEFGHIJK-Solid Support
C23456789012$BCDEFGHIJKL-Solid Support
C23456789012$CDEFGHIJKLM-Solid Support
... and so on.

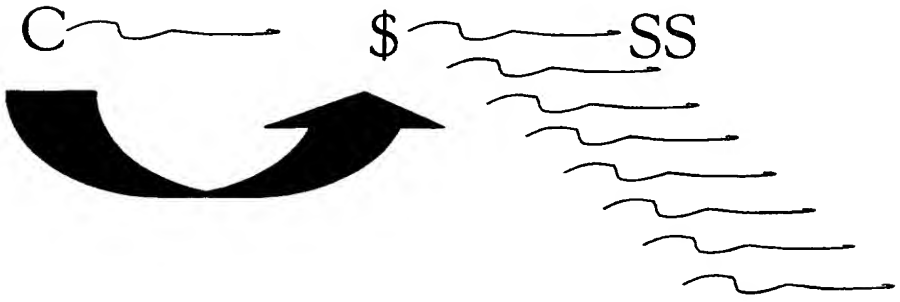
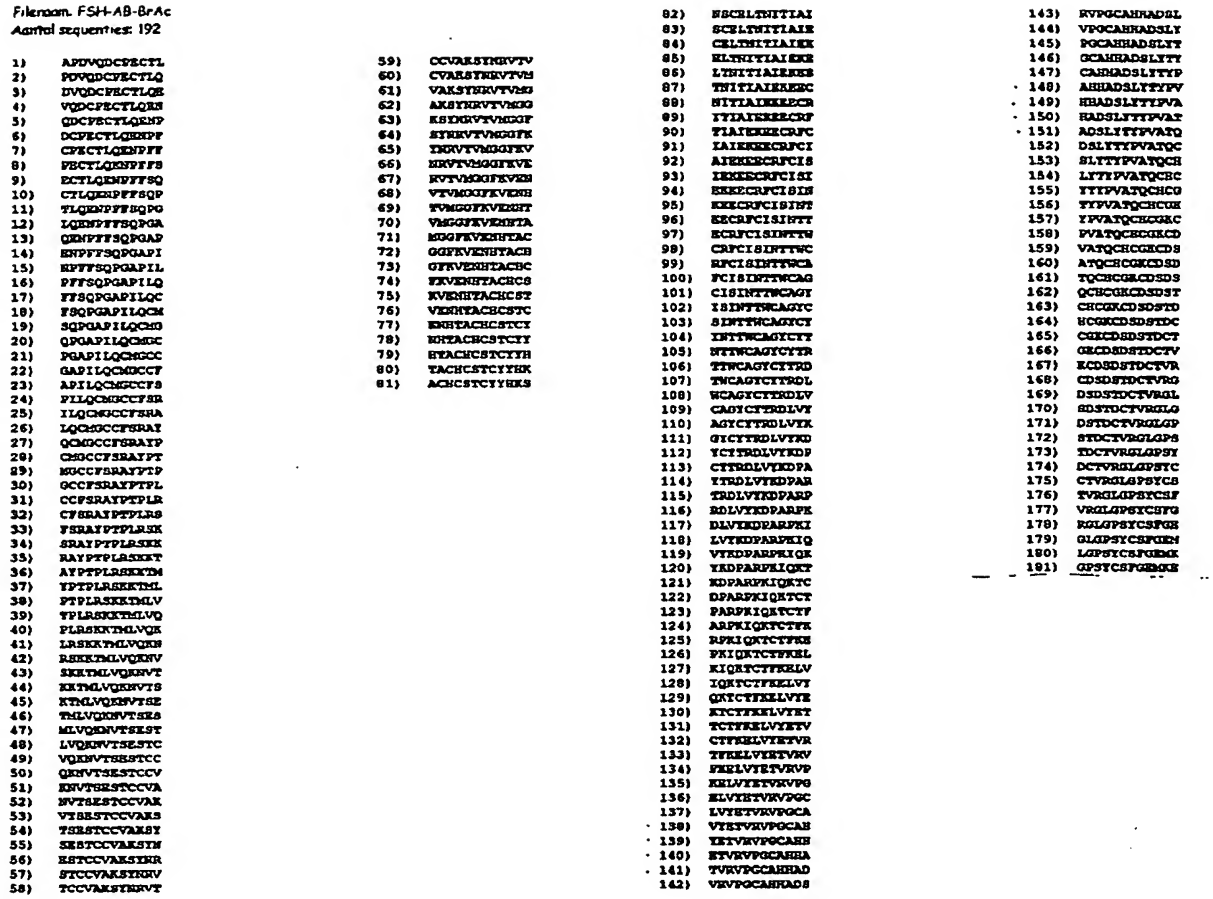


Fig. 6A.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4) VQDCPECTLQES
5) QDCPECTLQESP
6) DCPECTLQESPF
7) CPECTLQESPFF
8) PECTLQESPFFS
9) ECTLQESPFFSQ
10) CTLQESPFFSQP
11) TLQESPFFSQPG
12) LQESPFFSQPGA
13) QESPFFSQPGAP
14) ESPFFSQPGAPI
15) SPFFSQPGAPIL
16) PFFSQPGAPILQ
17) FFSQPGAPILQC
18) FSQPGAPILQCM
19) SQPGAPILQCMG
20) QPGAPILQCMGC
21) PGAPILQCMGCC
22) GAPILQCMGCCF
23) APILQCMGCCFS
24) PILQCMGCCFSR
25) ILQCMGCCFSRA
26) LQCMGCCFSRAI
27) QCMGCCFSRAIP
28) CMGCCFSRAIPT
29) MGCCFSRAIPTP
30) GCCFSRAIPTPL
31) CCFSRAIPTPLR
32) CFSRAIPTPLRS
33) FSRAIPTPLRSK
34) SRAIPTPLRSKK
35) RAIPTPLRSKKT
36) AIPTPLRSKKTM
37) IPTPLRSKKTML
38) PTPLRSKKTMLV
39) TPLRSKKTMLVQ
40) PLRSKKTMLVQK
41) LRSKKTMLVQKS
42) RSKKTMLVQKSV
43) SKKTMLVQKSVT
44) KKTMLVQKSVTS
45) KTMLVQKSVTSE
46) TMLVQKSVTSES
47) MLVQKSVTSESS
48) LVQKSVTSESTC
49) VQKSVTSESTCC
50) QKSVTSESTCCV
51) KSVTSESTCCVA
52) SVTSESTCCVAK
53) VTSESTCCVAKS
54) TSESTCCVAKSY
55) SESTCCVAKSYN
56) ESTCCVAKSYKR
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61) VAKSYKRVTVMG
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63) KSYKRVTVMGGF
64) SYKRVTVMGGFK
65) YKRVTVMGGFKV
66) KRVTVMGGFKVE
67) RVTVMGGFKVEN
68) VTVMGGFKVENH
69) TVMGGFKVENHT
70) VMGGFKVENHTA
71) MGGFKVENHTAC
72) GGFKVENHTACH
73) GFKVENHTACHC
74) FKVENHTACHCS
75) KVENHTACHCST
76) VENHTACHCSTC
77) ENHTACHCSTCY
78) NHTACHCSTCYY
79) HTACHCSTCYYH
80) TACHCSTCYYHK
81) ACHCSTCYYHKS
82) HSCHLTNITIAI
83) SCHLTNITIAIE
84) CHLTNITIAIEK
85) HLTNITIAIEKE
86) LTNITIAIEKES
87) TNITIAIEKESC
88) NITIAIEKESCR
89) ITIAIEKESCRF
90) TIAIEKESCRFC
91) IAIEKESCRFCI
92) AIEKESCRFCIS
93) IEKESCRFCISI
94) EKESCRFCISIS
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96) ESCRFCISISNT
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98) CRFCISISNTTW
99) RFCISISNTTWC
100) FCISISNTTWCA
101) CISISNTTWCAG
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103) SISNTTWCAGYC
104) ISNTTWCAGYCT
105) SNTTWCAGYCTT
106) NTTWCAGYCTTR
107) TTWCAGYCTTRD
108) TWCAGYCTTRDL
109) WCAGYCTTRDLV
110) CAGYCTTRDLVY
111) AGYCTTRDLVYK
112) GYCTTRDLVYKD
113) YCTTRDLVYKDP
114) CTTRDLVYKDPA
115) TTRDLVYKDPAR
116) TRDLVYKDPARP
117) RDLVYKDPARPK
118) DLVYKDPARPKI
119) LVYKDPARPKIQ
120) VYKDPARPKIQK
121) YKDPARPKIQKT
122) KDPARPKIQKTC
123) DPARPKIQKTCT
124) PARPKIQKTCTF
125) ARPKIQKTCTFK
126) RPKIQKTCTFKE
127) PKIQKTCTFKEL
128) KIQKTCTFKELV
129) IQKTCTFKELVT
130) QKTCTFKELVTE
131) KTCTFKELVTET
132) TCTFKELVTETV
133) CTFKELVTETVR
134) TFKELVTETVRV
135) FKELVTETVRVP
136) KELVTETVRVPG
137) ELVTETVRVPGC
138) LVTETVRVPGCA
139) VTETVRVPGCAH
140) TETVRVPGCAHH
141) ETVRVPGCAHHA
142) TVRVPGCAHHAD
143) RVPGCAHHADSL
144) VPGCAHHADSLY
145) PGCAHHADSLYT
146) GCAHHADSLYTT
147) CAHHADSLYTTP
148) AHHADSLYTTPV
149) HHADSLYTTPVA
150) HADSLYTTPVAT
151) ADSLYTTPVATQ
152) DSLYTTPVATQC
153) SLYTTPVATQCH
154) LYTTPVATQCHC
155) YTTPVATQCHCG
156) TTPVATQCHCGK
157) TPVATQCHCGKC
158) PVATQCHCGKCD
159) VATQCHCGKCDS
160) ATQCHCGKCDSD
161) TQCHCGKCDSDS
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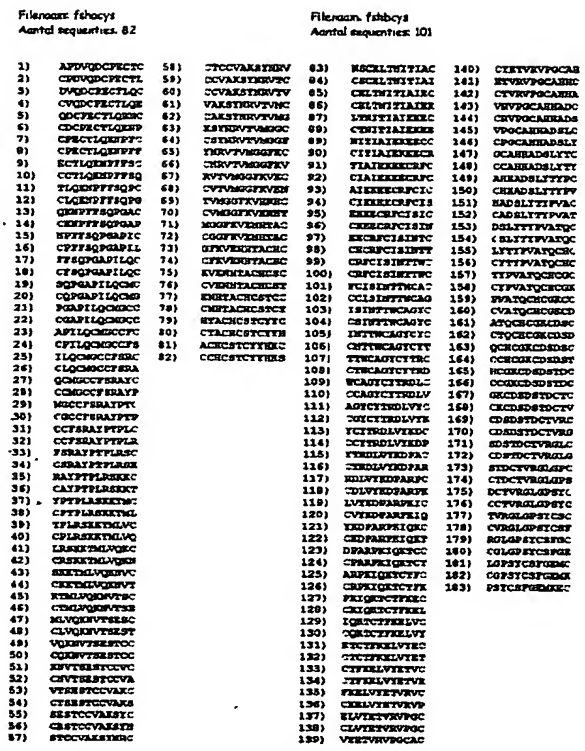
163) CHCGKCDSDSTD
164) HCGKCDSDSTDC
165) CGKCDSDSTDCT
166) GKCDSDSTDCTV
167) KCDSDSTDCTVR
168) CDSDSTDCTVRG
169) DSDSTDCTVRGL
170) SDSTDCTVRGLG
171) DSTDCTVRGLGP
172) STDCTVRGLGPS
173) TDCTVRGLGPSY
174) DCTVRGLGPSYC
175) CTVRGLGPSYCS
176) TVRGLGPSYCSF
177) VRGLGPSYCSFG
178) RGLGPSYCSFGE
179) GLGPSYCSFGEM
180) LGPSYCSFGEMK
191) GPSYCSFGEMKE

Fig. 6B.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Filenaam: fshbcys
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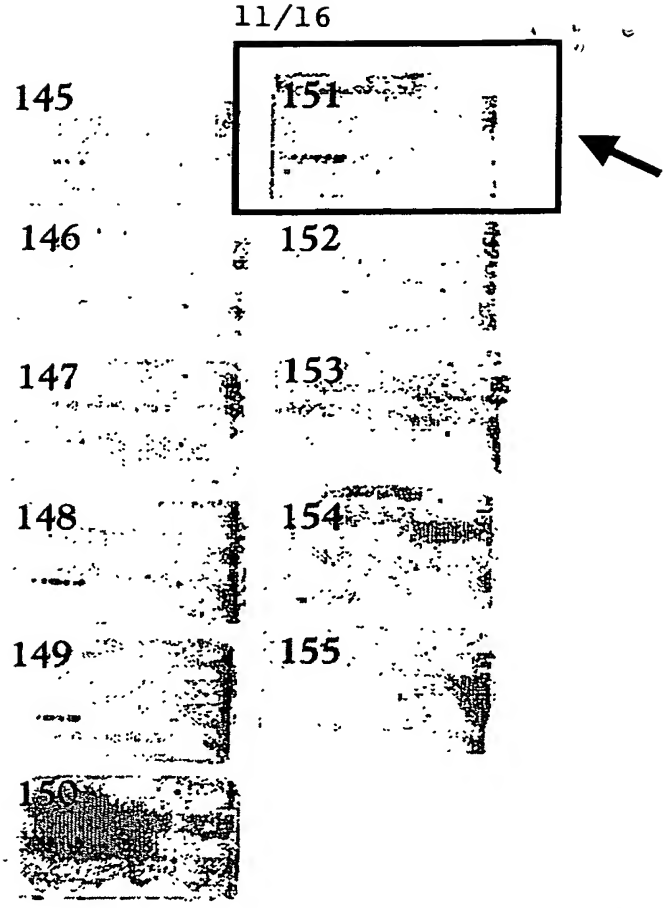
Fig. 6C.



Card 151, in detail

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31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	-
46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	-
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	-
76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	-
91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	-
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							143	144	145	146	147	148	149	150	181
151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	182
166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	183

Quantitive fluorescence-values:

136: 1308
137: 1793
138: 1586
139: 3276:VYETVRVPGCAC$ADSLYTYPVATQ
140: 2638
141: 2533
142:4038
background: 157

Total picture of ca. 40.000
25-mer peptides

Matrix-scan mAb-01 (5 ug/ml)

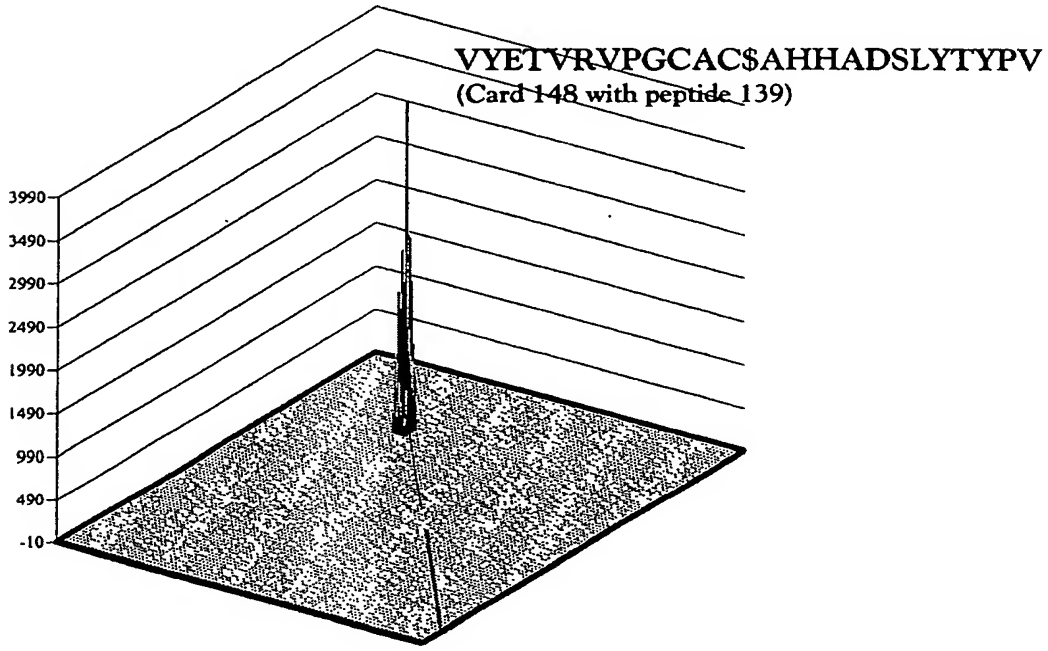

Fig. 6D.

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Solid Support-C$23456789012C$OPQRSTUVWXYZC$CDEFGHIJKLMN
Solid Support-C$34567890123C$PQRSTUVWXYZAC$DEFGHIJKLMNO
... and so on.

Fig. 7A.

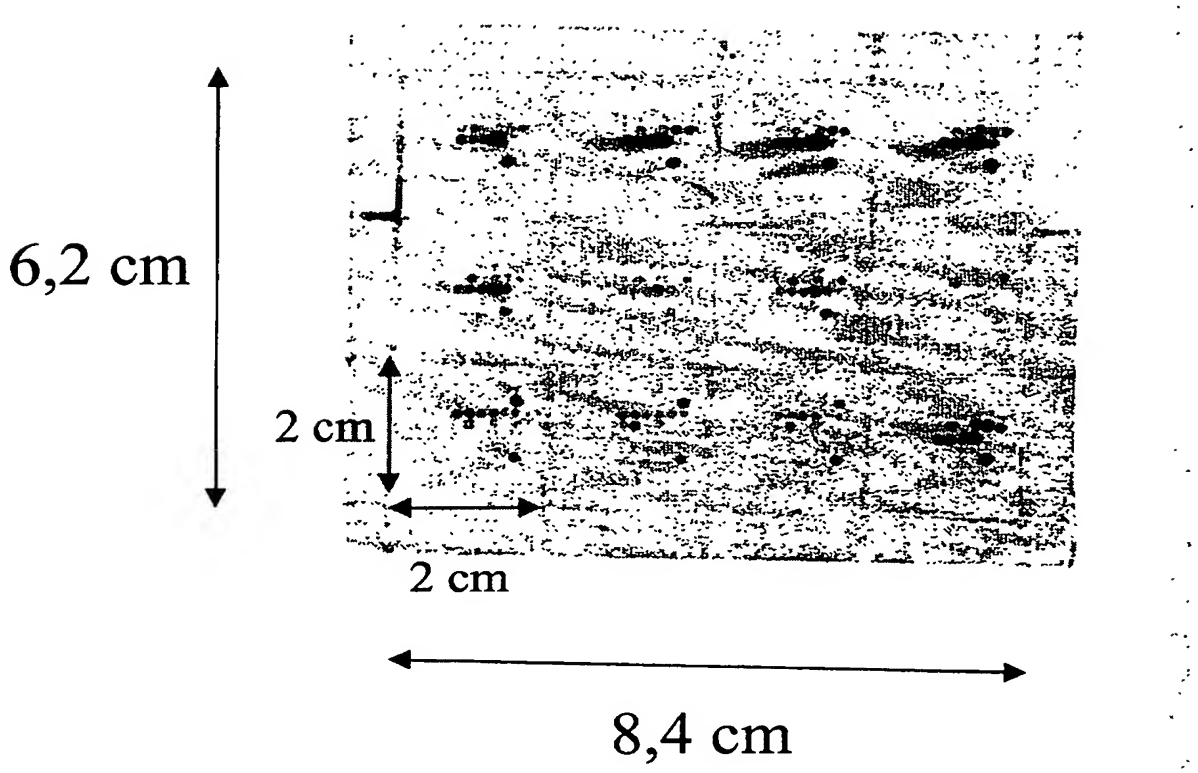
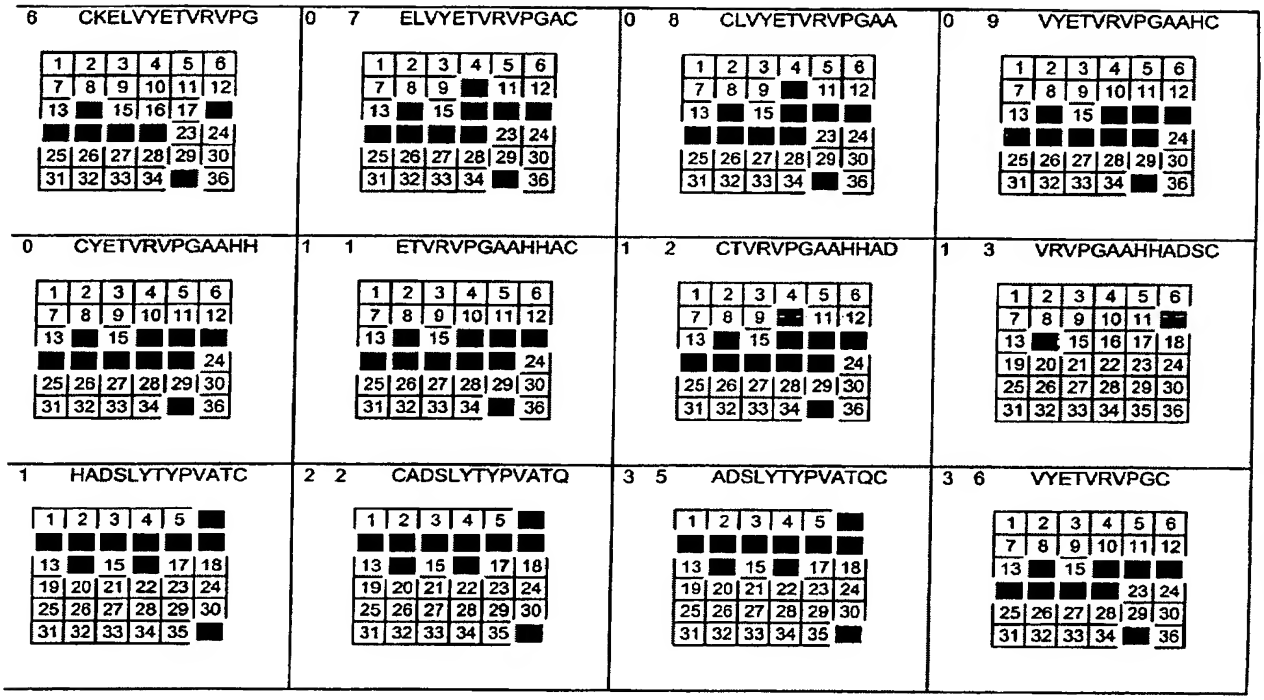
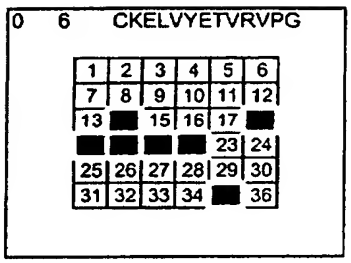


Fig. 7B.



1	KTATFKELVYETC
2	CTATFKELVYETV
3	ATFKELVYETVRC
4	CTFKELVYETVRV
5	FKELVYETVRVPC
6	CKELVYETVRVPG
7	ELVYETVRVPGAC
8	CLVYETVRVPGAA
9	VYETVRVPGAAHC
10	CYETVRVPGAAHH
11	ETVRVPGAAHHAC
12	CTVRVPGAAHHAD
13	VRVPGAAHHADSC
14	CRVPGAAHHADSL
15	VPGAAHHADSLYC
16	CPGAAHHADSLYT
17	GAAHHADSLYTYC
18	CAAHHADSLYTYP
19	AHHADSLYTYPVC
20	CHHADSLYTYPVA
21	HADSLYTYPVATC
22	CADSLYTYPVATQ
23	DSLYTYPVATQAC
24	CSLYTYPVATQAH
25	LYTYPVATQAHAC
26	CYTYPVATQAHAG
27	TYPVATQAHAGKC
28	CYPVATQAHAGKA
29	PVATQAHAGKADC
30	CVATQAHAGKADS
31	ATQAHAGKADSDC
32	CTQAHAGKADSDS
33	QAHAGKADSDSTC
34	CAHAGKADSDSTD
35	ADSLYTYPVATQC
36	VYETVRVPGC

Fig. 7C.



1	KTATFKELVYETC	107	1	107
2	CTATFKELVYETV	97	2	97
3	ATFKELVYETVRC	98	3	98
4	CTFKELVYETVRV	101	4	101
5	FKELVYETVRVPC	101	5	101
6	CKELVYETVRVPG	124	6	124
7	ELVYETVRVPGAC	107	7	107
8	CLVYETVRVPGAA	112	8	112
9	VYETVRVPGAAHC	121	9	121
10	CYETVRVPGAAHH	116	10	116
11	ETVRVPGAAHHAC	109	11	109
12	CTVRVPGAAHHAD	129	12	129
13	VRVPGAAHHADSC	125	13	125
14	CRVPGAAHHADSL	555	14	555
15	VPGAAHHADSLYC	380	15	380
16	CPGAAHHADSLYT	206	16	206
17	GAAHHADSLYTYC	184	17	184
18	CAAHHADSLYTYP	420	18	420
19	AHHADSLYTYPVC	1332	19	1332
20	CHHADSLYTYPVA	920	20	920
21	HADSLYTYPVATC	994	21	994
22	CADSLYTYPVATQ	1056	22	1056
23	DSLYTYPVATQAC	229	23	229
24	CSLYTYPVATQAH	101	24	101
25	LYTYPVATQAHAC	119	25	119
26	CYTYPVATQAHAG	124	26	124
27	TYPVATQAHAGKC	139	27	139
28	CYPVATQAHAGKA	147	28	147
29	PVATQAHAGKADC	143	29	143
30	CVATQAHAGKADS	150	30	150
31	ATQAHAGKADSDC	115	31	115
32	CTQAHAGKADSDS	111	32	111
33	QAHAGKADSDSTC	130	33	130
34	CAHAGKADSDSTD	143	34	143
35	ADSLYTYPVATQC	1047	35	1047
36	VYETVRVPGC	197	36	197

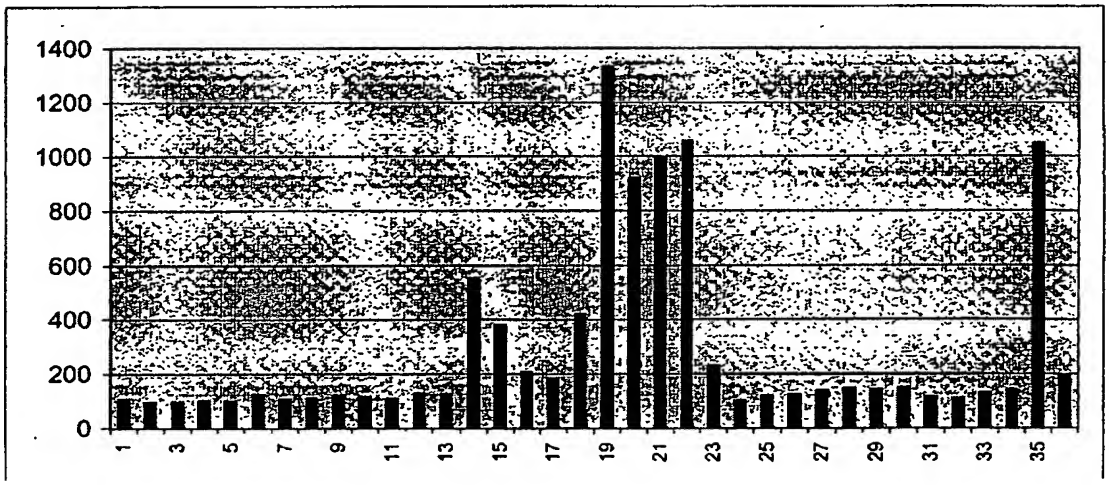


Fig. 7D.

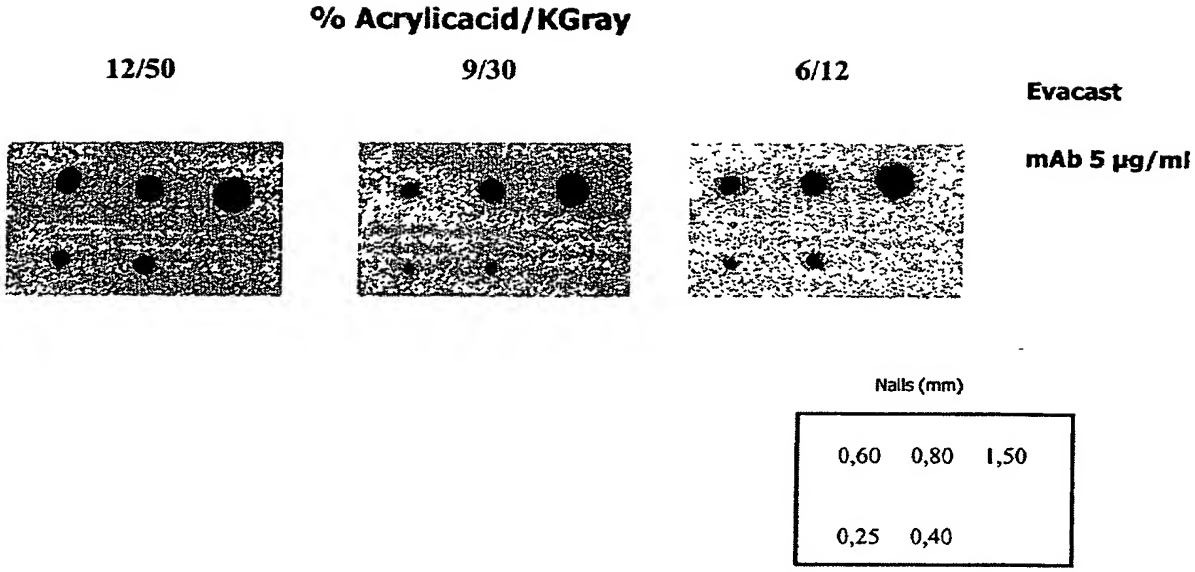

% Acrylicacid/KGray
12/50
9/30
6/12
Evacast
mAb 5 µg/ml
Nails (mm)
0,60
0,80
1,50
0,25
0,40

Fig. 8.